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Simultaneous determination of seven major isosteroidal alkaloids in bulbs of *Fritillaria* by gas chromatography

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Abstract

The present paper describes the development of a most simple, sensitive, and specific gas chromatographic method to date, for the direct determination of seven major bioactive isosteroidal alkaloids, namely ebeiedine, ebeiedinone, ebeienine, hupehenine, isoverticine, verticine, verticinone and imperialine, in *Fritillaria* species, a commonly used antitussive traditional Chinese medicinal (TCM) herb. In the present study, a commercially available Supelco SAC-5 capillary column (30 m×0.25 mm, 0.25 μm) specifically designed for the analysis of steroids was utilized for the direct determination of *Fritillaria* alkaloids. Calibration curves were obtained by spiking authentic compounds and the internal standard (solanidine) into herbal samples prior to extraction. Extraction was conducted simply by shaking the pre-alkalized diethyl ether solution (5.0 ml) containing dried herb (0.1 g) for 2 h. All calibration curves showed good linear regressions (r^2>0.995) within test ranges. The assay was reproducible and accurate with the overall intra- and inter-day variation and accuracy of less than 10% and more than 90%, respectively. The developed GC method was successfully utilized to analyze seven major bioactive alkaloids in seven *Fritillaria* species, and the results demonstrate that this direct GC analytical method is suitable for the quality control of this commonly used antitussive TCM herb.

Keywords: *Fritillaria* spp.; Alkaloids

1. Introduction

In Chinese communities worldwide the most widely used antitussive and expectorant traditional Chinese medicinal (TCM) herb is bulbus *Fritillariae* (Chinese name Beimu), which is derived from the bulbs of various species of the genus *Fritillaria* (*Liliaceae*) [1]. The chemical constituents present in Beimu have been extensively investigated, and eight isosteroidal alkaloids (Fig. 1) were established to be the major biologically active components in different *Fritillaria* species used as TCM herbs [2–7]. However, the amount and type of isosteroidal alkaloids present in various *Fritillaria* species can be different and subsequently, clinical outcomes can also vary with different usefulness of *Fritillaria* spp. Therefore, the development of simple and appropriate quality control methods for both qualitative and quantitative determination of the major active com-

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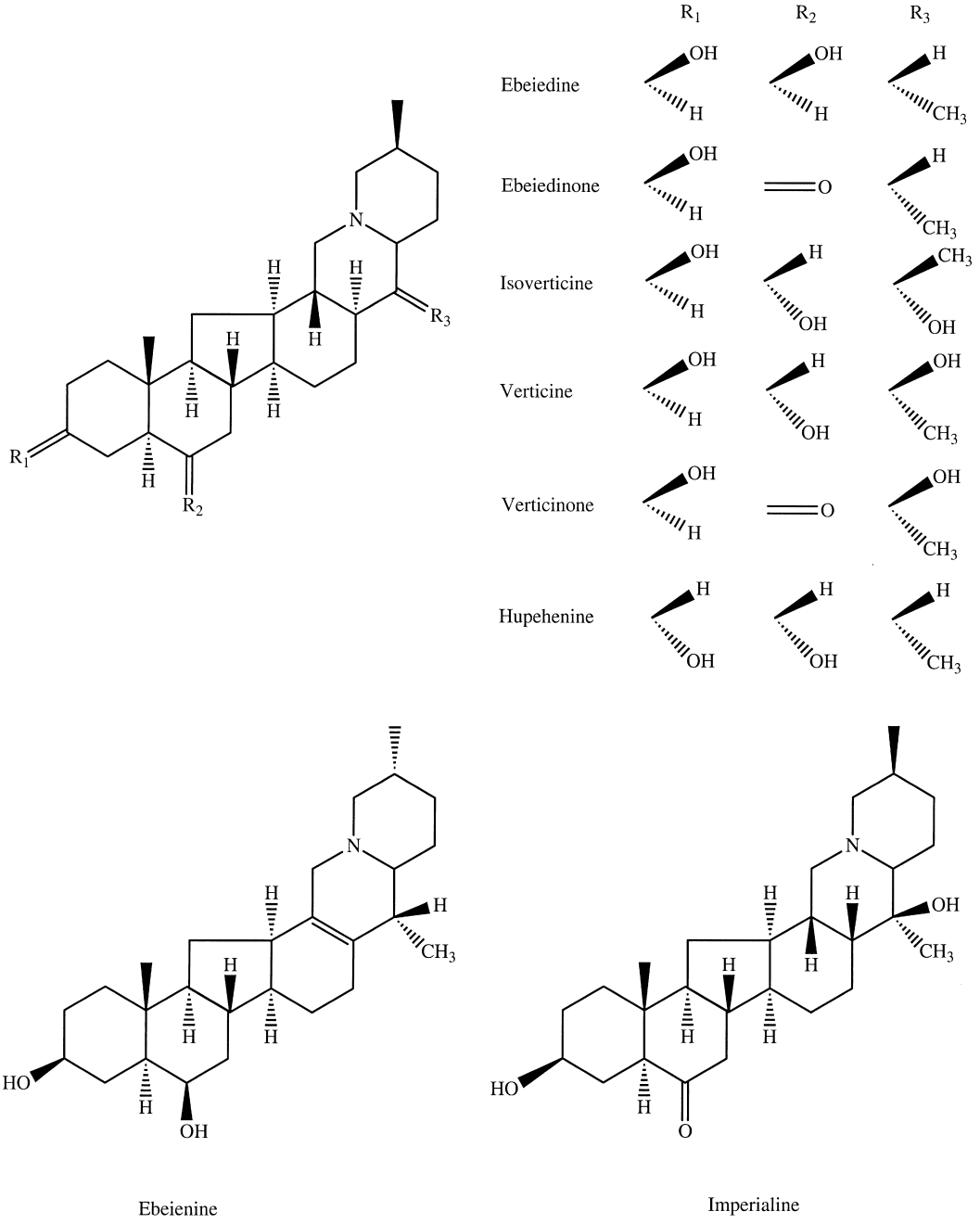

Fig. 1. Structures of ebeiedine, ebeiedinone, ebeienine, hupehenine, isoverticine, verticine, verticinone and imperialine.

ponents in Beimu has long been a challenge to scientists studying this TCM herb [8–11].

Previously, only a few HPLC analytical methods have been developed for the analysis of isosteroidal alkaloids [12], including the pre-column derivatization HPLC analytical assays for five major *Fritillaria* alkaloids developed by our research team [13]. The main difficulty of analysis is due to a lack of

ultraviolet absorption in most of the isosteroidal alkaloids. Recently we have established a GC analytical method for the simultaneous determination of five major isosteroidal alkaloids. However, as the alkaloids tested are highly polar and cannot be eluted from conventional GC columns, a pre-column derivatization process was required prior to GC analysis [14]. In order to simplify the assay, recently our research team conducted further investigations of different GC columns, and has demonstrated that using Supelco SAC-5 capillary column, a specific GC column for the analysis of steroids, all major active *Fritillaria* alkaloids can be directly eluted without pre-column derivatization. In this study, the newly developed GC method with direct analysis of seven isosteroidal alkaloids present in various *Fritillaria* species is described.

2. Experimental

2.1. Chemicals and materials

Solanidine and organic solvents with analytical reagent grade were all purchased from Sigma (St. Louis MO, USA). Eight isosteroidal alkaloids: ebeiedine, ebeiedinone, ebeienine, hupehenine, isoverticine, verticine, verticinone and imperialine were isolated from various *Fritillaria* spp. in our laboratories [3,11,15–21]. The purity of the isolated alkaloids were shown to be higher than 99% analyzed by HPLC, and their identities were confirmed by IR, ^{1}H- and ^{13}C-NMR, and MS analyses. Seven *Fritillaria* spp. were purchased in local TCM shops in China and authenticated by Dr. P. Li. The voucher specimens were deposited in the Department of Pharmacognosy, China Pharmaceutical University.

2.2. Apparatus and chromatographic conditions

GC analysis was performed using HP5890 series 2 gas chromatograph (Hewlett-Packard) equipped with a flame ionization detection (FID) system. Data were recorded and analyzed by HP3396 series 2 integrator. A Supelco SAC-5 capillary column (30 m$\times$ 0.25 mm, 0.25 μm) was utilized. Nitrogen was used as the carrier gas at a flow-rate of 30 cm/s. The oven temperature was operated at 295°C, and both injector and detector temperatures were set at 310°C. An aliquot (6 μl) of sample was injected with a split injection of 100:1.

2.3. Calibration curves

Dichloromethane stock solutions containing ebeiedine, ebeiedinone, ebeienine, hupehenine, isoverticine, verticine and imperialine were prepared and then diluted to appropriate concentration ranges for the construction of calibration curves. Each calibration curve was performed in triplicate with five different concentrations. The concentration of the internal standard, solanidine, was 10 μg/ml for all analyses. Calibration curves were constructed by spiking authentic analytes and the internal standard into the pre-alkalized (ammonium hydroxide) diethyl ether solution (5.0 ml), which contained powders of *F. cirrhosa* (0.1 g), prior to extraction. The resultant mixtures were then extracted as described in Section 2.6. Aliquots (6 μl) of the extracts were directly analyzed by GC. For the control samples, extracts of *F. cirrhosa* spiked with the internal standard only were prepared and analyzed in the same manner. Calibration curves were derived by plotting concentrations of each analyte as a function of peak area ratio differences (peak area ratio$_{\text{spiked}}$−peak area ratio$_{\text{control}}$) between spiked and control extracts.

2.4. Accuracy and precision

The measurements of intra- and inter-day variability were utilized to determine the accuracy and precision of the developed assay. Known quantities of seven analytes and the internal standard were added to the alkalized diethyl ether solution (5.0 ml) containing *F. cirrhosa* (0.1 g) prior to extraction. Control samples spiked with the internal standard only were also prepared similarly. The resultant samples were extracted and analyzed as described in Section 2.6. The peak area ratio difference between testing and control samples for each analyte was calculated, and the quantity of each analyte was subsequently obtained from the corresponding calibration curve. Each sample was analyzed in triplicate to determine the intra-day variability. The relative standard deviation (RSD) was taken as a measure of precision and the percentage difference between

amounts determined and spiked was considered as a measure of accuracy. The inter-day reproducibility was examined in three separate days.

2.5. Limits of detection

Aliquots of seven analytes were spiked into the alkalized diethyl ether solution (5.0 ml) containing *F. cirrhosa* (0.1 g) to provide a concentration range of 0.1–1.0 μg/ml. The resultant mixtures were extracted and analyzed in the same manner as described in Section 2.6. The limit of detection for each analyte was determined when the ratio of peak area of the analyte to noise was greater than five.

2.6. Analysis of seven alkaloids in Fritillaria species

To the grounded dried Beimu samples (0.1–0.2 g, adjusted according to the alkaloids contents of each *Fritillaria* species), 5.0 ml of diethyl ether pre-alkalized with ammonium hydroxide and 50 μl of the internal standard solution (1 mg/ml) were added. The mixtures were shaken by vortex for 2 h and then centrifuged at 1780 *g* for 10 min. The supernatants (2.5 ml) were transferred into vials and evaporated to dryness. The obtained residues were reconstituted into 100 μl of dichloromethane, and aliquots (6 μl) of the resultant extracts were directly subjected to GC analysis. The contents of the analytes were determined from the corresponding calibration curves.

3. Results and discussion

As shown in Fig. 1, all alkaloids tested contain at least one polar hydroxyl functional group and cannot be eluted from conventional GC columns, thus only a GC analytical method with pre-column derivatization has been previously established by our research team [14]. Obviously the inclusion of pre-column derivatization has disadvantages. It is more time consuming and requires completion of the derivatization in order to achieve accuracy of quantification. Therefore, the aim of the present study was to develop a direct GC analytical method for the analysis of *Fritillaria* alkaloids. Two commercially available capillary columns, namely Supelco SAC-5 (30 m×0.25 mm, 0.25 μm) specifically designed for the analysis of steroids, and HP-1 (12.5 m×0.22 mm, 0.33 μm) for compounds having hydroxyl groups were examined. It was found that using the Supelco SAC-5 column, six alkaloids and the internal standard were resolved well with base-line separations (Fig. 2A), although verticine and verticinone co-eluted under the various conditions examined. In the case of using HP-1 column, peak tailings were observed for most of the alkaloids tested, and verticine and verticinone could not be separated. Therefore, the Supelco SAC-5 column was chosen for the study. Six of eight *Fritillaria* alkaloids tested were separately quantified, while contents of verticine and verticinone were determined as a sum of these two alkaloids.

Blank controls are generally unavailable for the study of herbal materials, and thus calibration curves are normally conducted without using the internal standard method. Therefore, reproducibility and extraction yield become critical for quantification of the principal components in herbs. In order to solve this problem, our research team had recently developed an internal standard method [22]. Briefly, both internal standard and analytes tested were spiked into the herbal samples prior to extraction, while for the control, only the internal standard was added separately to similar herbal samples prior to extraction. Calibration curves were then constructed as a function of the concentration of analyte versus the peak area ratio differences between spiked and non-spiked (control) herbal extracts. This internal standard method was also adopted in the present study. Since verticine and verticinone could not be separated under the developed GC conditions, the total content of these two alkaloids present in herbs was determined from the calibration curve for verticine. As summarized in Table 1 the seven calibration curves all showed good linear regressions. Furthermore, the results demonstrated that the developed direct GC analytical method is reproducible with good accuracy. The overall intra- and inter-day variations were less than 10% for all analytes, and the overall intra- and inter-day accuracy for the determination of all alkaloids tested were higher than 91% and 90%, respectively (Table 2). In addition, the developed assay provided good sensitivity for all analytes. The

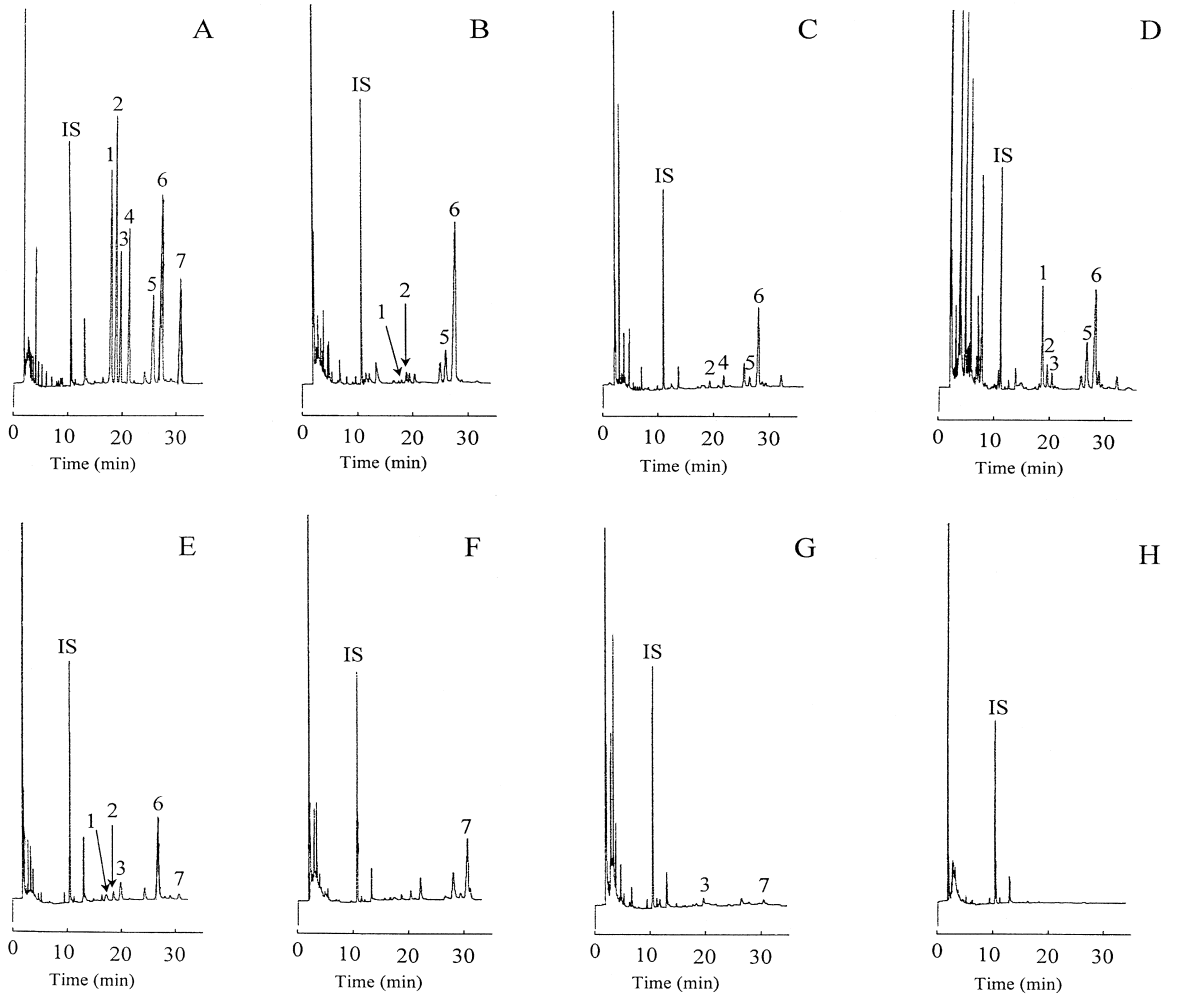

Fig. 2. Representative GC chromatograms of the extracts of: (A) *F. cirrhosa* spiked with standards; (B) *F. thunbergii*; (C) *F. hupehensis*; (D) *F. ebeiensis*; (E) *F. cirrhosa*; (F) *F. pallidiflora*; (G) *F. ussuriensis*; and (H) *F. maximowiczii*. IS, internal standard; 1, ebeiedine; 2, ebeiedinone; 3, ebeienine; 4, hupehenine; 5, isoverticine; 6, verticine and verticinone; 7, imperialine.

Table 1
Calibration curves for seven *Fritillaria* alkaloids

Alkaloid	Retention time (min)	Standard curve[a]	r^2	Test rang (μg/ml)	Test limit (μg/ml)
Ebeiedine	18.01	$y=0.0497x-0.0883$	0.997	1.6–46.8	0.52
Ebeiedinone	18.96	$y=0.0583x-0.1013$	0.996	1.6–46.6	0.52
Ebeienine	19.76	$y=0.0441x-0.0499$	0.996	0.9–27.3	0.30
Hupehenine	21.34	$y=0.0514x-0.0652$	0.995	1.0–31.3	0.35
Isoverticine	25.87	$y=0.0353x-0.0578$	0.995	1.3–39.8	0.44
Verticine	27.44	$y=0.0374x-0.1397$	0.996	3.1–93.5	0.50
Imperialine	30.87	$y=0.0437x-0.0673$	0.995	1.3–38.6	0.43

[a] y: Difference of peak area ratio (peak area ratio$_{spiked}$ − peak area ratio$_{control}$); x: concentration of analyte (μg/ml).

Table 2
Intra- and inter-day variability for the assay of seven *Fritillaria* alkaloids

Alkaloid added (μg/ml)	Intra-day variability			Inter-day variability		
	Detected (n=3)	RSD (%)[a]	Accuracy (%)[b]	Detected (n=3)	RSD (%)[a]	Accuracy (%)[b]
Ebeiedine						
7.79	7.75±0.23	3.0	96.9	7.64±0.19	2.5	98.1
15.58	14.23±0.56	3.9	91.5	14.12±0.49	3.5	90.6
31.16	29.93±2.88	9.6	96.1	29.71±3.08	10.4	95.3
Ebeiedinone						
7.77	7.80±0.91	2.4	99.6	7.82±0.19	2.4	99.4
15.53	14.57±0.40	2.7	93.8	14.52±0.37	2.5	93.5
31.06	28.97±0.29	1.0	93.3	29.24±0.69	2.3	94.1
Ebeienine						
4.55	4.56±0.06	1.3	99.8	4.55±0.06	1.4	100.0
9.10	8.51±0.21	2.4	93.5	8.51±0.26	3.1	93.5
18.20	16.89±0.37	2.2	92.8	16.84±0.40	2.4	92.5
Hupehenine						
5.22	5.22±0.13	2.6	100.0	5.26±0.11	2.0	99.2
10.44	9.75±0.35	3.6	93.4	9.76±0.33	3.3	93.5
20.88	19.27±0.07	0.4	92.3	19.22±0.31	1.6	92.0
Isoverticine						
6.63	6.60±0.16	2.5	99.5	6.70±0.11	1.6	98.9
13.25	12.34±0.42	3.4	93.1	12.36±0.29	2.4	93.3
26.50	24.52±0.42	1.7	92.5	23.76±1.32	5.5	89.7
Verticine						
15.58	15.65±0.35	2.3	99.6	15.78±0.29	1.8	98.7
31.15	29.35±1.25	4.2	94.2	29.15±0.79	2.7	93.6
62.30	57.50±0.74	1.3	92.3	57.66±1.66	2.9	92.6
Imperialine						
6.44	6.51±0.17	2.6	98.9	6.49±0.14	2.1	99.2
12.88	12.14±0.46	3.8	94.3	12.12±0.21	1.7	94.1
25.75	23.57±0.12	0.5	91.5	23.90±0.65	2.7	92.8

[a] RSD (%) (relative standard deviation)=(SD/mean)×100.
[b] Accuracy (%)=[1−(mean concentration measured−concentration spiked)/concentration spiked]×100.

detection limits were 26.0 μg/g of the dried herb for ebeiedine and ebeiedinone, 25.0 μg/g for verticine and verticinone, 15.0 μg/g for ebeienine, 17.5 μg/g for hupehenine, 22.0 μg/g for isoverticine and 21.5 μg/g for imperialine, respectively.

The developed GC assay was subsequently applied to the simultaneous determination of seven major isosteroidal alkaloids in seven Beimu samples. Representative chromatograms of the extracts of different *Fritillaria* spp. are shown in Fig. 2, and the quantity of each alkaloid identified in seven herbs are summarized in Table 3. The results demonstrated that the developed direct GC analysis is compatible with our previously established GC method with pre-column derivatization [14]. Among seven *Fritillaria* spp. selected for the present study, four species namely *F. thunbergii*, *F. cirrhosa, F. pallidiflora* and *F. ussuriensis* are documented in the China pharmacopoeia as the plant sources for herbal Beimu [23]. The other three are only used locally depending

Table 3
Contents of isosteroidal alkaloids in various *Fritillaria* species[a]

Fritillaria spp.	Content (μg/g)						
	Ebeiedine	Ebeiedinone	Ebeienine	Hupehenine	Isoverticine	Verticine and Verticinone	Imperialine
F. cirrhosa Don	46.65±3.38	43.21±2.40	82.75±7.13	nd	nd	272.00±20.54	34.18±0.98
F. pallidiflora Schrenk	nd	nd	nd	nd	nd	nd	307.76±14.51
F. ussuriensis Manim	nd	nd	nd	nd	nd	nd	37.09±3.61
F. thunbergii Miq.	tr	87.31±9.32	nd	nd	276.33±19.56	1313.69±115.07	nd
F. hupehensis Hsiao *et* Hasi	tr	tr	nd	152.79±15.27	181.59±14.24	1208.99±75.15	nd
F. ebeiensis Yu *et* Ji Var. *purpurea* Yu *et* Li	137.14±2.47	117.14±7.80	104.95±7.86	nd	750.71±23.22	1337.06±119.46	nd
F. maximowiczii Freyn	nd	nd	nd	nd	nd	nd	nd

[a] tr: Content of the alkaloid was lower than the test limit and could not be quantified. nd: not detected.

on the locations of their growth. It was interesting to note that with the exception of *F. thunbergii*, imperialine, which is the most potent antitussive *Fritillaria* alkaloid [7], was found in three *Fritillaria* spp. documented in the China pharmacopoeia, but was not detected in any of the three locally used Beimu samples (Table 3). Furthermore, hupehenine was identified only in *F. hupehensis*, which has been reported to be the most toxic *F.* species used clinically [8,9]. In addition, none of seven alkaloids tested were determined in *F. maximowiczii.* Therefore, although it needs further clarification, variations of the types and quantities of the major active *Fritillaria* alkaloids present in different *Fritillaria* species may be responsible for the different usefulness and therapeutic outcomes of these TCM herbs. Investigations into the correlation between biologically active components present in different Beimu species and their pharmacological activities are currently under progress in our research laboratories.

4. Conclusions

In conclusion, to date, the presently developed direct GC method for the analysis of seven major active *Fritillaria* alkaloids is the simplest quantitative method for the determination of the major active components in Beimu, the most commonly used antitussive TCM herb. This GC analytical assay is sensitive, accurate, and reproducible. It provides a suitable quality control method for Beimu samples and can be readily utilized for the determination of the major active ingredients present in this herb.

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